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PRINCIPAL INVESTIGATOR: Michael S. Kinch, Ph.D.

CONTRACTING ORGANIZATION: Purdue University  
West Lafayette, Indiana 47907-1063

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13. ABSTRACT ( <i>Maximum 200 Words</i> )  The EphA2 receptor tyrosine kinase functions differently in normal and malignant epithelial cells. The goal of the present project was to determine if EphA2 recognizes different substrates in non-transformed and transformed human mammary epithelial cells. To ask this, we utilized a new approach of generating "unnatural" ATP specificity through site-specific mutation of the EphA2 kinase domain. Although these mutants were successfully generated and expressed in non-transformed and malignant epithelial cells, the EphA2 in these cells was unable to distinguish between standard and "unnatural." Consequently, this experimental approach was not able to identify the substrates recognized by EphA2 in normal and malignant cells. Current studies are seeking to identify the reasons why EphA2 fails to distinguish between standard and "unnatural" ATP as this information could be useful in understanding the regulation of EphA2 enzymatic activity in breast cancer.			
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**INTRODUCTION:**

EphA2 is a receptor tyrosine kinase that functions very differently in non-transformed versus transformed mammary epithelial cells <sup>1;2</sup>. In non-transformed epithelia, stable cell-cell adhesions allow EphA2 to bind its ligands, which are anchored to the cell surface, and become tyrosine phosphorylated <sup>3</sup>. In malignant cells, unstable cell-cell adhesions prevent ligand binding <sup>2</sup>. Unlike other receptor tyrosine kinases, EphA2 enzymatic activity does not require ligand binding <sup>2</sup>. Rather, the phosphotyrosine content of EphA2 directs its subcellular localization and protein interactions. Whereas unphosphorylated EphA2 promotes tumor cell growth and migration, tyrosine phosphorylated EphA2 inhibits tumor cell growth and migration <sup>1;2</sup>. In our approved Concept Award, we had hypothesized that EphA2 recognizes different substrates in normal and malignant mammary cells. As a proof-of-principle study, we proposed to identify EphA2 substrates using a new approach. In collaboration with Dr. Kevan Shokat at the University of California at San Francisco, we sought to generate a mutant that would allow EphA2 to accept “unnatural” forms of radiolabeled ATP <sup>4</sup>. We proposed to express this mutant in cells that lack EphA2 and to identify its radiolabeled substrates by SDS-PAGE and autoradiography. We then sought to treat the cells with an artificial form of ligand, EprinA1-Fc, and ask if the substrate profile of EphA2 differs when it is phosphorylated versus unphosphorylated EphA2. We had intended prioritize the identification of substrates that are unique to either normal or malignant cells, since these would be most relevant to the regulation of breast tumorigenesis. These pilot studies were intended to lay the groundwork for follow-up studies to identify the substrates and to determine how EphA2-mediated phosphorylation of these substrates regulates breast epithelial cell growth and migration.

**BODY:***Generation of Mutant EphA2:*

A thorough analysis of the kinase domain of EphA2 revealed a threonine residue at position 692 that was analogous to the site where Dr. Kevan Shokat had previously generated mutant kinases with “unnatural” ATP specificity. Dr. Ming Lu, a new post-doctoral fellow in the Kinch laboratory using PCR-based mutagenesis, mutated this residue into either an alanine or glycine. The successful generation of these mutations were then confirmed by sequencing of the EphA2 cytoplasmic domain.

*Expression and Analysis of EphA2 Mutants:*

To analyze the EphA2 mutants, each was overexpressed in two different mammary epithelial cell lines, MCF-7 and MCF-10A. Both were selected because they express E-cadherin. Since we had previously demonstrated that E-cadherin allows for tyrosine phosphorylation of EphA2 and that E-cadherin function can be easily manipulated in these systems<sup>2</sup>, we felt that these systems offered an opportunity to evaluate the potential effects of EphA2 autophosphorylation upon substrate specificity. Moreover, the systems differed in that MCF-7 has very low levels of endogenous EphA2 whereas MCF-10A cells do express relatively high levels of EphA2.

Our initial studies sought to overexpress EphA2 in MCF-7 cells. This was accomplished using both transient and stable transfection assays. The expression of transfected EphA2 was confirmed by Western blot analyses with specific antibodies and by flow cytometry. We then utilized in vitro kinase assays to ask if the transfected EphA2 could demonstrate enzymatic activity using <sup>32</sup>P-labeled ATP. As expected, the wild-type EphA2 demonstrated robust autophosphorylation activity. Although the mutant forms of the molecule retained some intrinsic enzymatic, mutation of residue 692 dramatically decreased enzymatic activity. This mutant demonstrated decreased autophosphorylation activity and was completely unable to phosphorylated exogenous substrates (e.g., enolase). In large part, the decrease in enzymatic activity was attributable to relatively low levels of expression. Multiple and different experiments all confirmed that the mutants of residue 692 were poorly expressed. It is generally understood that mis-folded receptor tyrosine kinases are frequently degraded by chaperones and that this can prevent adequate overexpression. We believe that this mechanism was responsible for the lower levels of residue 692 mutants because EphA2 mRNA levels were consistently overexpressed whereas a much weaker overexpression of protein was detected.

An analogous experiment was attempted using MCF-10A cells. However, these assays mostly detected endogenous EphA2 as compared with the transfected mutant. Despite the lower levels of enzymatic activity, we elected to proceed based on the low level of enzymatic activity detected using MCF-7 cells.

### *“Unnatural” Enzymatic Activity*

The wild-type and mutant forms of EphA2 were then tested for their abilities to utilize “unnatural” forms of ATP. We obtained multiple and different forms of ATP from our collaborator, Dr Kevan Shokat, and asked if these could prevent EphA2 from utilizing standard  $^{32}\text{P}$ -labeled ATP as measured using standard ATP competition assays. Indeed, the “unnatural” ATP molecules were able to block the residue 692 mutants as measured using in vitro kinase assays with immunoprecipitated material. Unfortunately, control studies revealed that these “unnatural” forms of ATP were also capable of inhibiting the enzymatic activity of wild-type EphA2. We ruled out that this outcome had resulted from a mis-labeling of the wild-type plasmid by confirming the inhibitory activity of ATP analogs upon endogenous EphA2 that was expressed in MCF-10A cells.

After multiple attempts to obtain selectivity, we considered that the “unnatural” ATP might have been contaminated with standard ATP and contacted our collaborator. In response, Dr. Kevan Shokat supplied us with different preparations of the inhibitors but the outcomes were again disappointing. We were unable to achieve selective inhibition of the mutant kinase relative to wild-type EphA2. Dr. Shokat was very helpful in our experimentation but eventually conceded that this was the first kinase in which his experimental strategy had not been successful.

The next step in our studies would have been to directly conjugate the “unnatural” ATP to radioactive phosphorous  $^{32}\text{P}$ . However, given the hazards of this procedure, along with our lack of specificity as detailed above, we did not feel that it would be appropriate to continue with this line of investigation.

### KEY RESEARCH ACCOMPLISHMENTS:

- Generation of EphA2 T692 mutants
- Stable and transient overexpression of the T692 mutants in MCF-7 and MCF-10A cells
- Kinase assays to evaluate enzymatic activity
- Assessment of “unnatural” ATP specificity

**REPORTABLE OUTCOMES:**

- Development of MCF-7 cells that overexpress wild-type or mutant (A692 or G692) EphA2

**CONCLUSIONS:**

The studies conducted to date suggest a number of issues that prevent an analysis of EphA2 substrates using the experiment approach of "unnatural" ATP utilization. First, mutation of residue 692 appears to prevent EphA2 overexpression relative to wild-type EphA2. This reduction in EphA2 levels greatly hindered our ability to analyze the biochemical or biological activities of the mutant EphA2. It is likely that the mutant EphA2 is mis-folded and thus degraded by cellular regulatory mechanisms associated with chaperones. Second, wild-type EphA2 appears to utilize "unnatural" forms of ATP. This differs from reports of other kinases, in which the wild-type forms of the enzyme do not utilize the ATP analogues. We are presently performing x-ray crystallography to ask if this lack of selectivity is related to structural motifs surrounding the kinase domain.

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**APPENDICES:**

None